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FINAL **REPORT ON**

CONTRACT NO DA 92-557-FEC-33136

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SUBJECT OF INVESTIGATION

EXPLANATION OF PATHOGENESIS OF

JAPANESE B ENCEPHALITIS

AND

ESTABLISHMENT OF AVIRULENT STRAINS OF

JAPANESE B ENCEPHALITIS VIRUS

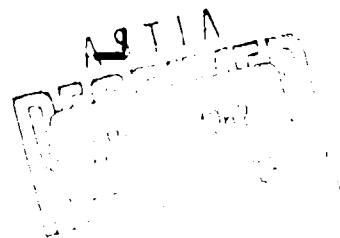
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National Institute of Health, Tokyo (Japan)
EXPLANATION OF PATHOGENESIS OF JAPANESE B ENCEPHALITIS AND ESTABLISHMENT OF AVIRULENT STRAINS OF JAPANESE B ENCEPHALITIS VIRUS by Akira Oya.
Final rept No. 2, 21 p. Incl. illus. tables, 9 refs.

(Contract DA 92-557-FEC-33136) Unclassified report
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EXPLANATION OF PATHOGENESIS OF JAPANESE B ENCEPHALITIS

AND

ESTABLISHMENT OF AVIRULENT STRAINS OF

JAPANESE B ENCEPHALITIS VIRUS

PART II

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1. VARIATION OF PERIPHERAL INFECTIVITY OF JAPANESE B ENCEPHALITIS VIRUS STRAINS TO ADULT MICE.

a. Purpose of Study

Japanese B encephalitis (JBE) virus infects a variety of mammals including man in nature. A large part of infected animals has been found to result in inapparent infection and only a few individual seems to manifest encephalitic symptoms. This divergent pathogenicity is considered to be influenced by at least two factors, property of the virus and condition of the host. The purpose of our present study is to investigate pathogenicity from virus side.

Since our study described in the previous final report has suggested some variation of peripheral infectivity to adult mice among JBE strains, more detailed survey of the variation has been made with a number of JBE strains on our present hand.

b. Materials and Methods

Virus strains: A number of JBE strains maintained in our laboratory has been employed. They have a variety of isolation and passage histories. Details are described on the corresponding tables. Virus materials employed in the experiment are infected baby and adult mouse brains and infected tissue culture fluid.

Virus diluent: Phosphate buffered saline at pH 7.4 containing heated horse or calf or rabbit serum was used. Serum had been confirmed to be free of JBE antibody.

Mice: For suckling mice, dd or RFVL strain was employed at their 2 to 4 days after birth. Both strains were confirmed to show the same susceptibility to JBE strains when inoculated i.c.. As the adult mice, 28 to 32 day old gpc strain mice propagated at the Department of Veterinary Science of our institute were used exclusively. Those mice were fed with the commercial dried food.

Test for peripheral virulence to adult mice: Virus materials were diluted tenfoldly. Two tenth cc was inoculated i.p. into each of 5 adult mice with a dilution routinely. At the same time, 0.02 cc was inoculated i.c. into each of a litter suckling mice with the same dilution so as to determine the infective titer of the original materials. Titer was shown as BLU (baby mouse lethal unit) in \log_{10} . Infection was judged from the death and the presence of HI antibody of the survived adult mice 3 weeks after the inoculation. Virulence of the virus strain was determined from the results of infection of adult mice considering the infective titers obtained by suckling mice.

Hemagglutination inhibition test: The antigen was prepared with the same strain which had been employed for the virulence test to avoid any immunological strain difference. HI technique followed that described by Clarke and Casals (1958).

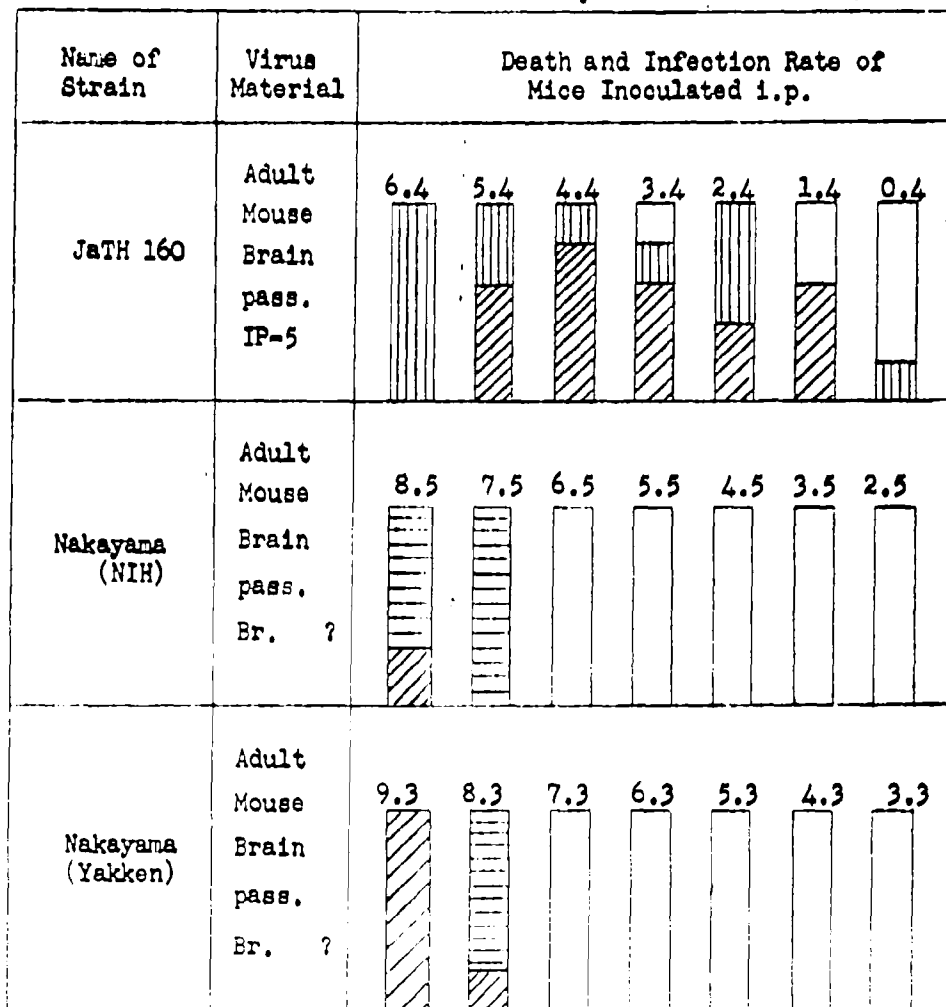
Isolation of viral clones: Clones were isolated from plaques obtained on chick embryo monolayer. Plaque technique was essentially the same to that described by Inoue et al (1961).




c. Experimental Results

- 1) Three distinct type of peripheral infectivity of JBE strain to adult mice.

As described in the previous report, a newly isolated JBE strain JaTH 160 killed adult mice frequently even when a few BLU was injected intraperitoneally. Some of inoculated mice were found to survive for the observation period of 3 weeks with or without showing symptoms. Antibody to JBE virus could be demonstrated with sera of these surviving mice suggesting the occurrence of infection. On the contrary, no adult mice died in case of a laboratory fixed strain, Nakayama-NIH when less than $10^{7.0}$ BLU was injected i.p.. Low titer antibody was spottedly found in the adult mice. In other words, the former strain seemed to have strong infectivity while the latter had very weak one to the adult mice by the peripheral injection. The results are demonstrated in Fig. 1.

Fig. 1. Peripheral infectivity test of a newly isolated and laboratory-fixed JBE strains.

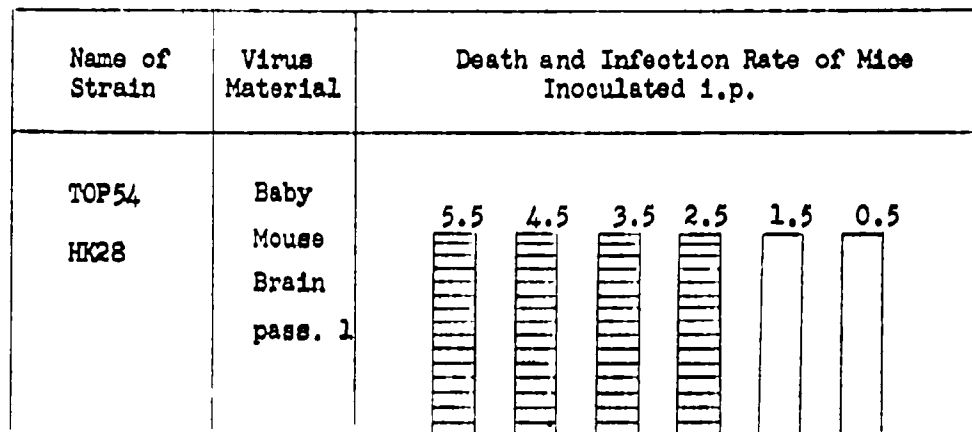


Note:  Death  Antibody detected individually
 Antibody detected with pooled sera

Numbers on top of the column denote BLU injected.

It is assumed that there may be strains bearing an intermediate peripheral infectivity to adult mice. It may no more kill adult mouse but retain the infectivity stimulating antibody response. Such property has been eventually found with a strain derived from our partially attenuated TOP strain of JBE virus. As indicated in Fig. 2, it no more kill adult mouse by the peripheral inoculation but still retain a property to immunize mice.

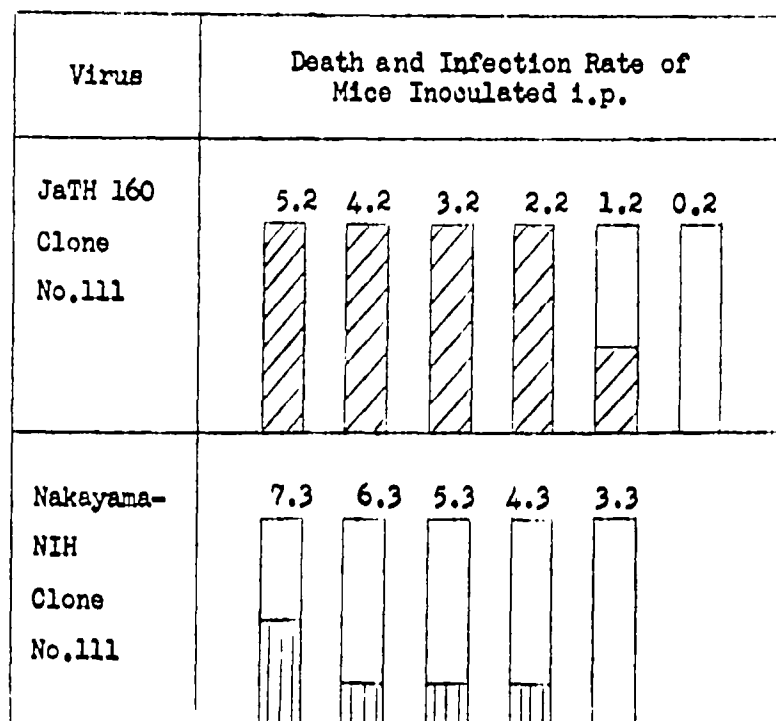
Fig. 2. Peripheral infectivity of a strain driven from the attenuated TOP strain



Note: The same to Fig. 1

A question may reasonably arise that a strain of virus consists of a mixed population of virus particles of different properties. The experimental facts observed above may be due, in part, to such a mixed character. In order to deny this possibility, viral clones were separated by means of plaque isolation technique from JaTH 160 and Nakayama-NIH. Peripheral infectivity was reexamined with these clones. The results will be seen in Fig. 3.

Fig. 3. Peripheral infectivity of some cloned virus



Note: The same to Fig. 1.

The difference of the results was clearly confirmed in the level of clone suggesting genetic control to the peripheral infectivity. Finally, one can postulate that there are at least three types of JBE virus concerning its peripheral infectivity to adult mouse, lethal, non-lethal but infective and poorly infective.

2) Classification of current JBE strains according to their virulence types.

The experimental results described above revealed the evidences to differentiate JBE strains according to their peripheral infectivity to adult mice. There are a number of JBE strains available to the test in our laboratory. These strains have a variety of isolation and passage history. An attempt was made to classify those strains for their peripheral infectivity to adult mice. The method employed here was described in Materials and Methods.

The criteria to determine their types were made as follows:

Lethal type (L^+I^+); More than one mouse died by the i.p. inoculation of less than $10^{7.0}$ BLU.

Non lethal but infective type (L^-I^+); No mouse died by the i.p. inoculation of less than 10^7 BLU, and consistent HI antibody was shown with the sera of surviving mice 3 weeks after the inoculation of less than $10^{7.0}$ BLU.

Non infective type (L⁻I⁻); No death and no consistent HI production was observed in mice inoculated with less than 10⁷ BLU.

The results are listed on Table 1.

Table 1. Classification of peripheral infectivity type of JBE strains

Name of Strain	Sources of Isolation	Method of Isolation	*Passages	Materials Employed	Type of Peripheral Infectivity
JaTH 160	Human Brain	AMIP-Br	AMIP-Br(9)	AMBr	L ⁺ I ⁺
JaTH 260	" "	-	-	Human Brain	L ⁺ I ⁺
KVI	" "	BMBR	BMBR(3)	BMBR	L ⁺ I ⁺
Tanaka	" "	AMBr	AMBr(4)	AMBr	L ⁺ I ⁺
Igaya	" "	AMBr	AMBr(3)BMBR(2)	BMBR	L ⁺ I ⁺
Kitano	" "	BMBR	BMBR(1)AMBr(25)BMBR(1)	BMBR	L ⁺ I ⁺
G-1	" "	AMBr	AMBr(7)BMBR(4)	BMBR	L ⁺ I ⁺
Nakayama-RFVL	" "	AMBr	AMBr(7)BMBR(11)	BMBR	L ⁺ I ⁺
JaGAR 01	Mosquito	HK	HK(8)	HKFL	L ⁺ I ⁺
JaGAR 02	"	BMBR	BMBR(3)	BMBR	L ⁺ I ⁺
JaGAR 15460	"	BMBR	BMBR(2)OE(20)BMBR(1)	BMBR	L ⁺ I ⁺
Nakayama-NIH	Human Brain	AMBr	AMBr(733)BMBR(1)	BMBR	L ⁻ I ⁻
Nakayama-Yakken	" "	AMBr	AMBr(790)BMBR(1)	BMBR	L ⁻ I ⁻
G-1	" "	AMBr	AMBr(195)BMBR(1)	BMBR	L ⁻ I ⁻
I - 58	" "	AMBr	AMBr(18)BMBR(6)	BMBR	L ⁻ I ⁻
Hotta	" "	AMBr	AMBr(4)BMBR(1)	BMBR	L ⁻ I ⁻
Calinina	" "	AMBr	AMBr(62)BMBR(1)	BMBR	L ⁻ I ⁻
2869	Horse Brain	AMBr	AMBr(35)		L ⁻ I ⁻
TOP-54HK6	Human Brain	OE	OE(54)HK(6)	HKFL	L ⁻ I ⁻
Cl-108					
TOP-54HK28	Human Brain	OE	OE(54)HK(28)BMBR(1)	BMBR	L ⁺ I ⁺

*AMBr.... Adult mouse brain BMBR....Baby mouse brain
 OE.... One day egg HK....Hamster kidney cells
 AMIP-Br....Adult mouse IP-Brain passage
 HKFL.... Hamster kidney culture fluid
 Number in parenthesis means number of passages.

It was felt that there were many grades between two extremes, a strongly lethal type and a completely non infective type. However, general trend could be observed that they were divided into 3 groups.

d. Discussion

Some experimental criteria were shown to differentiate JBE strains for their peripheral infectivity to adult mice.

However, one should notice that these criteria might only be applied to our specified strain of mice. Because, no other strain of mice had been examined yet. There may be many factors to influence such a viral virulence to mice, i.e. age, food, sanitary condition and other environmental conditions. However, as far as our repeated experiments indicated, classified types were found to be fairly reproducible.

As suggested by the results indicated on Table 1, new isolates seems to belong L⁺I⁺ type and strains of repeated laboratory passages have a trend to belong L⁻I⁻ type. In other words, L⁺I⁺ type may be thought to be "original" feature of JBE virus and following the routine adult mouse passages it may lose an ability to infect adult mouse by the peripheral inoculation and thus change to have L⁻I⁻ property.

The hypothesis would be supported by the results with a strain G-1, with which a material at earlier passage demonstrated L⁺I⁺ character and the one at late passage showed L⁻I⁻ character (See Table 1).

Passages through suckling mouse brain or ip-brain passages may help to maintain the original property. However, several attempts to change L⁺I⁺ virus strain to L⁻I⁻ by repeated adult mouse brain passage was not successful.

Most peculiar findings were obtained with Nakayama strain. By repeated experiment, two lines of Nakayama strain, one maintained in our laboratory and another supplied from Dr. Fujie, National Veterinary Assay Laboratory were found to belong L⁻I⁻ type. While third line of Nakayama supplied from The Rockefeller Foundation Virus Laboratories was shown definitely to belong L⁺I⁺ type.

A possibility can be considered that Japan strain deviated much by many passages, while RFVL strain still retain its original character either by fewer passages or by better condition to maintain virus.

Finally, there is a good reason to select a suitable strain to be employed for the study of pathogenesis of JBE virus in the laboratory.

e. Summary

The experimental evidences were shown to indicate difference of peripheral infectivity to adult mice of various JBE strains.

According to our criteria experimentally settled, there seem to be 3 types of the peripheral infectivity of JBE strain.

Following those criteria, a number of JBE strains of different isolation and passage history were classified for their virulence types. To study pathogenesis of JBE virus, it is advised to use a suitable strain after the careful examination of its peripheral infectivity.

2. STUDIES ON PATHOGENESIS OF PERIPHERAL INFECTION TO ADULT MICE WITH JAPANESE B ENCEPHALITIS VIRUS.

a. Purpose of Study

Experimental study hitherto obtained has elucidated three distinct types of peripheral infectivity of JBE virus. The present report deals with the results of further study which attempted to analyse factors affecting the infection and fate of virus particles inoculated peripherally into adult mouse.

b. Materials and Methods

Cortizone acetate: Aqueous suspension of cortone, Merck & Co., Inc. was used.

Treatment of mice with cortizone: Appropriate concentration of cortizone in PBS was administered s.c. daily 5 to 10 times during the early stage of infection. Total amount of cortizone was 0.1-0.2 mg/g body weight.

Immune serum: Hyperimmune mouse serum was prepared by repeated i.p. injection of inactivated and living JaTH 160 virus suspension. Infected mouse brain was used as an immunizing material.

c. Experimental Results

- 1) Two sorts of death of adult mice inoculated with JBE virus peripherally.

When a L^+ strain of JBE virus was inoculated i.p. into adult mice, death occurred even by the inoculation with a few BLU. Contrary to this, no death of mouse was found in case of injection with L^- strain except when a considerably large amount of virus, i.e. more than 10^7 BLU was applied. The fatal outcome caused by larger virus dosis seemed to be different from that caused by smaller dosis of L^+ strain by the following reasons. Death by larger dosis occurs earlier beginning 4th to 5th day of inoculation and ceases within a few days, while death by smaller dosis begins to occur at 7th day or later and lasts for more than a week. Further, former death can be seen both with L^+ and L^- strain, on the other hand, in the latter, death is observed only with L^+ strain. The difference of the incubation period suggests that earlier common death may result from a sort of direct inoculation of virus into brain though the inoculation was practically made i.p.. Death restricted to L^+ may mean the existence of some indirect mechanism mediated by the primary viral growth somewhere outside of brain.

- 2) Effect of cortizone upon the mortality of adult mice inoculated with L^+ virus.

As reported in the previous report, an attempt was made to select a strong L^+ variant by in-brain passages of JaTH 160 strain to adult mice. However, 100 per cent mortality was hardly observed in the mice inoculated with a good amount of virus selected by this procedure though surviving mice demonstrated antibody indicating inapparent infection. A possibility was examined whether some immune mechanism operating soon after the initiation of virus growth may play a role for mice to resist the infection. Thus, cortizone was applied to

interfere the immune mechanism. A group of mice was injected with cortizone daily for 5 days, 0.2 mg per gram body weight in total, starting from a day prior to the virus inoculation. Table 2 shows the mortality of mice treated with or without cortizone during the infection of L⁺ strain.

Table 2. Effect of cortizone upon JaTH 160 infection to adult mouse

Treatment	Mortality of mice inoculated with the following BLU dosis i.p.					
	# 4.9	3.9	2.9	1.9	0.9	1.9
None	*2/5	4/5	2/5	3/5	3/5	0/5
Cortizone	5/5	5/5	4/5	5/5	4/5	0/5

Note: # Number of mice died/Number of mice inoculated

* Expressed in log₁₀

As a consequence, mortality appeared to increase by cortizone treatment. However, the result was not reproduced by the fact that such a treatment of mice with cortizone alone was found frequently to kill the mice. An effort was made to find a optimal treatment of adult mouse with cortizone and it was advised to divide the cortizone injection 10 times or 5 times for the administration of 0.2 mg or 0.1 mg in total.

Nevertheless, when those new technique was applied, no difference resulted in the mortality of mice inoculated with L⁺ virus with or without cortizone treatment.

At last, no conclusion was obtained with those experiments.

3) Age factor of mice employed for the experiment of peripheral infectivity.

As already pointed out by Lennette and Koprowski (1944), susceptibility of mice to peripheral inoculation with JBE virus were thought to vary depending upon their ages. Accordingly, it was examined with our inbred strain of mice infected with L⁺ and L⁻ strain of JBE virus. It is clear, as seen on Table 3, that there is a marked change in susceptibility of mice between 2 and 3 week age. It was in quite a coincidence to the results reported by the previous investigators. However, it is also elucidated that there is a marked difference on virulence between L⁺ and L⁻ strains of JBE virus.

Table 3. Age difference of mice concerning their susceptibility to JBE virus inoculated i.p.

Strain of virus	Inocula (*BLU)	Per cent of mortality of infected mice at various ages			
		2w	3w	4w	5w
JaTH 160	1.5	**71	20	60	0
	2.5	86	100	80	20
	3.5	89	20	80	20
	4.5	100	60	60	20
	5.5	100	100	40	40
Nakayama	1.7	0	0	0	0
	2.7	0	0	0	0
	3.7	22	0	0	0
	4.7	0	0	0	0
	5.7	20	0	0	0

Note: *Expressed as \log_{10} .

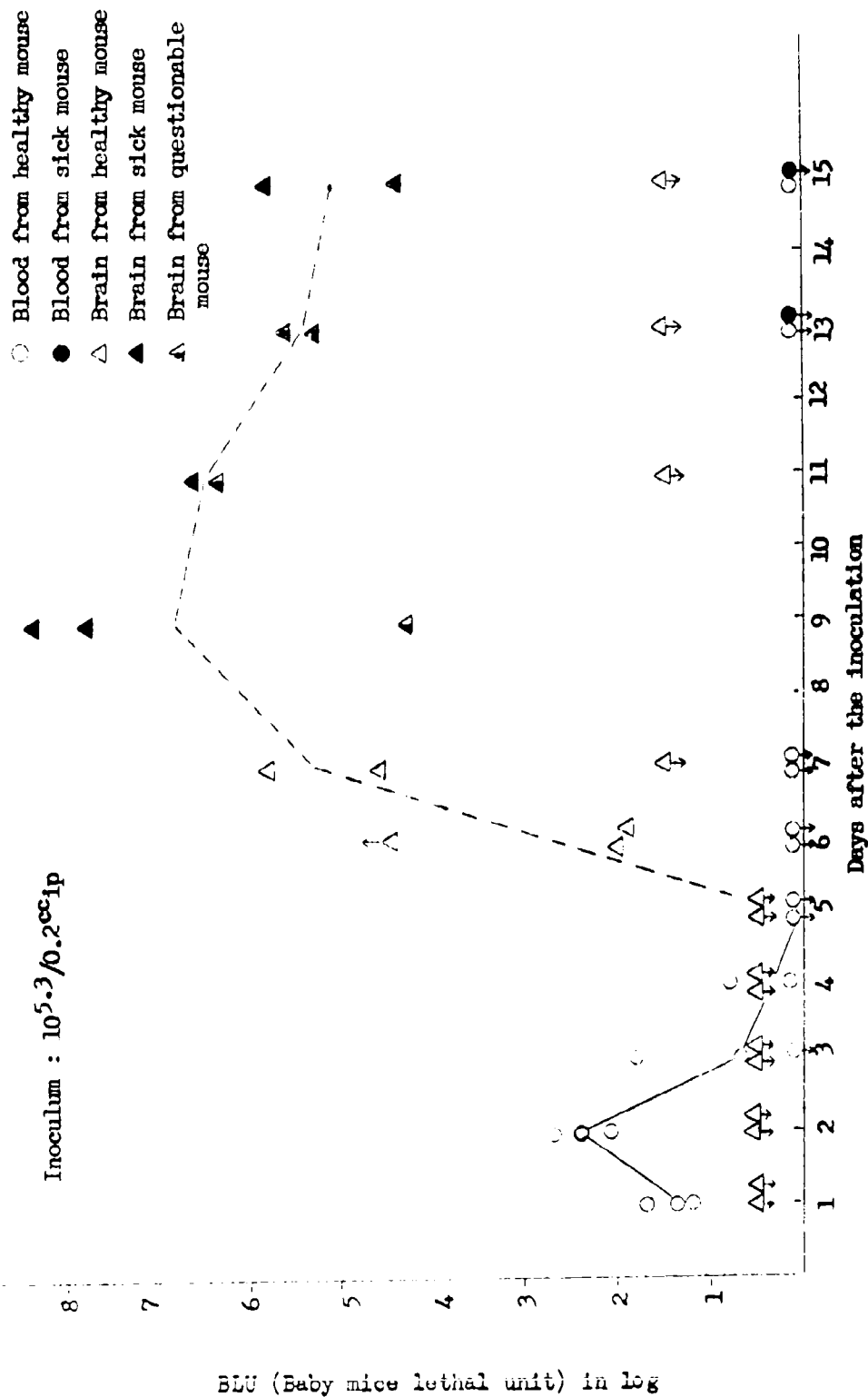
**Mortality per cent of mice inoculated.

4) Multiplication patterns of JBE virus in the adult mice inoculated intraperitoneally.

The long incubation period needed to observe death of peripherally infected adult mouse with L⁺ strain suggested the existence of primary virus multiplication somewhere outside of brain before the initiation of viral growth in brain tissue. To elucidate these sequences, the infective titre was followed in blood and brain of the adult mouse after the intraperitoneal inoculation of L⁺ strain.

Seventy adult mice were inoculated i.p. with $10^{5.3}$ BLU. Three mice randomly selected were sacrificed daily until 7th day, every other day from 7th to 15th day and their blood and brain samples were examined for infectivity content by i.e. titration with suckling mice. As expected, some of the mice manifested symptoms after 7th day and died leaving the other in apparently healthy condition. All surviving mice was found to have HI antibody at 3 weeks after the inoculation. Results are plotted in Fig. 4. It is clearly shown that there are two growth cycles, the first representing blood phase and the second brain phase. It is interesting to see that the viremia appeared inevitably, while viral multiplication in brain occurred all or none among three individuals. After 7th day, brains from sick mice contained a good amount of virus, while those from apparently normal mice yielded no active virus.

Fig. 4. Multiplication-pattern of JATH 160 strain inoculated intraperitoneally into 4 week old mice.



Then, a question may reasonably arise whether there is any difference of viral multiplication among L⁺I⁺, L⁻I⁻ and L⁻I⁺ strains. The same type of experiments were undertaken with an inoculum of 10^{4.3} and 10^{5.2} BLU per mouse for Nakayama-NIH (L⁻I⁻) and TOP54HK28 (L⁻I⁺) strains. No death occurred among mice of both groups during the observation period of 3 weeks. However, HI antibody was present only in the mice inoculated with L⁻I⁺ strain at the end of 3 weeks. The results of infectivity titration is shown on Table 4.

Table 4. Recovery of infective TOP54HK28EMI virus in adult mice inoculated i.p.

Organ	Mouse No.	Method to detect virus	Days after the inoculation (10 ^{5.2} BLU)											
			12h	1	2	3	4	5	6	7	9	11	13	15
Blood	1		-	-	+	-	-	-	-	-	-	-	-	-
	2	I.C. inoculation into suckling mice	-	-	+	+	-	-	-	-	-	-	-	-
	3		-	-	1/8	1/7	+	-	-	-	-	-	-	-
	4	HK cells	-	-	1/8	4/8	1/8	-	-	-	-	-	-	-
Brain	1	I.C. inoculation into suckling mice	-	-	4/4	1/4	2/4	-	-	-	-	-	-	-
	2		-	-	-	-	-	-	-	-	-	-	-	-
	3		-	-	-	-	-	-	-	-	-	-	-	-

Note: * Number of mice dead / Number of mice inoculated

** Number of HK tubes infected / Number of HK tubes inoculated

No active virus was recovered either from blood or from brain samples of mice inoculated with L⁻I⁻ strain. On the other hand, viremia was apparently demonstrated with mice inoculated with L⁻I⁺ strain, though its titer was so low. In the meantime, it was impossible to recover infective virus from mice of both groups.

Finally, it may be concluded that 3 JBE virus strains which are distinct each other with peripheral infectivity type have different mode of viral multiplication in adult mouse. In other words, different potency of JBE strains to multiply in adult mouse may cause the differences in their peripheral infectivity type.

5) Effect of immune serum upon viral growth in the adult mice inoculated with JBE virus peripherally.

The multiplication pattern of a L⁺I⁺JBE strain inoculated i.p. into adult mice suggested that viremia might be an essential event to cause viral infection in brain. Had it been the fact, it is considered to be possible to inhibit viral growth in brain by blocking viremia with the aid of immune serum in mice previously infected with a L⁺I⁺ JBE virus peripherally. Five groups of 2 week old mice were inoculated with various dose of JaTH 160 virus i.p.. At 0, 10, 24, 48 and 72 hrs of infection, 0.1 ml of immune mouse serum (HI titer: 1:1,280) was administered subcutaneously into a group of mice inoculated with various virus dose. In the separated experiment, 3 groups of 2 week old mice were inoculated intracerebrally with various virus dosis, then treated by the same lot of the immune serum at 0 and 10 hrs of infection. The last group was left as control. Mice were observed for 3 weeks to score deaths. Two week old mice were exclusively selected in these experiment, because mice were found to be highly susceptible at this age to the peripheral inoculation with L⁺I⁺ strain of JBE virus (Table 3). As shown in Table 5, the treatment by immune serum was found to be highly effective when it was injected until 10 hrs after the infection and the mice were infected by the peripheral route. On the other hand, little effect of the same treatment was observed when the mice were infected i.c.. These facts seem to support an idea that the immune serum administered at early infection stage is active to neutralize viremia so that the mice infected peripherally avoid viral invasion into brain tissue.

Table 5. Influence of administration of immune serum to young mice inoculated i.p. and i.c. with JaTH 160 strain

Route of infection	Inocula (*BLU)	Time of injection of immune serum after the infection			
		0	10 hrs	24 hrs	36 hrs
i.p.	5.3	**0	0	100	86
	4.3	0	11	100	100
	3.3	0	0	83	100
	2.3	0	0	29	50
i.c.	4.7	100	100	-	-
	3.7	100	100	-	-
	2.7	100	100	-	-
	1.7	83	90	-	-

Note: * Baby mouse i.c. lethal unit in log₁₀

** Mortality per cent of mice infected and treated by immune serum

d. Discussion

Several features observed during the experimental test of peripheral infectivity of JBE virus to mouse has been studied in the present research. As soon as we initiated the examination of mortality of adult mouse inoculated with JBE strains, it was noticed that two sorts of death of mouse were resulted following the viral inoculation. Our interest was focused to only one type of fatality of mice which ensued to the inoculation of lower inoculum. It could only be encountered with L⁺I⁺ strain of JBE virus. Further, the longer incubation period suggesting the preexistence of inapparent infection seemed to furnish an infection pattern similar to that occurring in nature. More detailed experiments to follow infective titer in blood and brain of mouse demonstrated a characteristic feature of L⁺I⁺ virus infection, dealing with viremia followed by virus growth in brain tissue.

However, some mice were found to be apparently normal during 3 weeks. No active virus was recovered after 7th day from such a healthy mouse. The fact suggested the existence of two different course of infection in adult mice peripherally infected with L⁺I⁺ strain. In the first one, viral multiplication occurs in brain causing illness to mouse following viremia. In the second, virus does not invade brain from blood vessels and the mouse remains apparently normal. On the other hand, an evidence was shown in the text that the application of immune serum could inhibit the death of mice at some extent when administered at an early stage of infection. This fact may be implicated by a mechanism that the immune serum neutralize viremia which eventually interfere the viral invasion into brain tissue. As a whole, one can separate a series of sequences occurring in the adult mouse peripherally infected with L⁺I⁺ virus as follows: viral multiplication outside of brain, viremia, viral invasion into brain tissue, viral multiplication in brain.

Diphasic growth of virus was encountered only with L⁺I⁺ strain, JaTH 160. Failure to recover in infective virus neither from blood nor from brain inoculated with L⁻I⁻ strain, Nakayama-NIH supported

an assumption that L⁻I⁻ virus can hardly multiply by peripheral injection. A strain derived from partially attenuated TOP strain showed only viremia without any indication of its growth in brain tissue. It is good evidence to classify the strain to L⁻I⁺ type concerning the peripheral infectivity. Consequently, three distinct types of JBE strain are considered to possess their own biological activities as tabulated on Table 6.

Table 6. Analytical schema of peripheral infectivity of JBE strain

Type of peripheral infectivity	Name of strain	Growth outside of brain	Induction of viremia	Invasion into brain	Growth in brain
L ⁺ I ⁺	JaTH 160	+	+	+	+
L ⁻ I ⁺	TOP54HK28	+	+	-	±
L ⁻ I ⁻	Nakayama-NIH	±	-	-	+

The activities of virus described above were only recognized when a standardized experimental condition was applied. An inbred strain of mouse was used throughout the experiment. Age factor of mouse played an important role to the test of peripheral infectivity of JBE virus. As indicated on Table 3, there was a marked difference in the susceptibility of mouse between 2 and 3 week of age. Four week old mice was exclusively employed for routine tests as the adult mouse.

e. Summary

To standardize the experimental condition for examining the peripheral infectivity of JBE strain, several host factors were analysed. Those were peculiar features of fatal outcome of infected adult mouse, the role of immune response on the course of infection and age factors of mouse to affect the results of infection. Finally, infective titers were followed in blood and brain of mouse peripherally infected with, L⁺I⁺, L⁻I⁺ and L⁻I⁻ strain of JBE virus. Resulted Growth curves seemed to add a good evidence for the classification concerning their virulence types.

3. ISOLATION OF THREE DISTINCT VIRUSES BEARING DIFFERENT VIRULENCES FROM A SINGLE STAIN OF JAPANESE B ENCEPHALITIS VIRUS.

a. Purpose of Study

There are two main purposes doing the present investigation. One is to follow probable variation of virulence of JBE virus during the passages in the laboratory. This kind of study was expected to reveal an experimental proof to the previous observation which demonstrated the existence of difference of virulence among JBE strains maintained in the laboratory. Another purpose is to isolate a JBE strain being able to produce immunity but completely a virulent to mouse when inoculated peripherally.

b. Materials and Methods

Virus: Tanaka strain of JBE virus, isolated from fatal brain of a patient in 1957 by Okuno (1959) was employed.

Standard passage of virus: When an infected brain was employed, 10% suspension was made with diluent, spun at 3,000 rpm for 10 min and supernatant was regarded as 10^{-1} dilution. 10^{-3} dilution was used as an inoculum. In case of tissue culture fluid, 10^{-2} dilution was used as an inoculum.

Terminal dilution passage of virus: In case of using one day old egg, refer Okuno's report (1959). When hamster kidney (HK) cell was applied, titration of virus was done using 4 tubes per dilution. Culture fluid of a tube showing clear cytopathic effect (CP) at the highest dilution of virus suspension was selected as a seed for the next titration-passage.

Undiluted passage of virus: Undiluted infected tissue culture fluid was inoculated 0.1 cc into 0.9 cc of maintenance fluid of a new HK tube. Four tubes were employed as routine.

Clone selection from virus suspension by HK tubes: Suitable dilution series of virus suspension were made in diluent and 30 to 32 HK tubes were inoculated with a dilution. Clonal virus suspension was taken from a tube showing CP at a dilution at which more than 75% of inoculated tubes were found CP-negative.

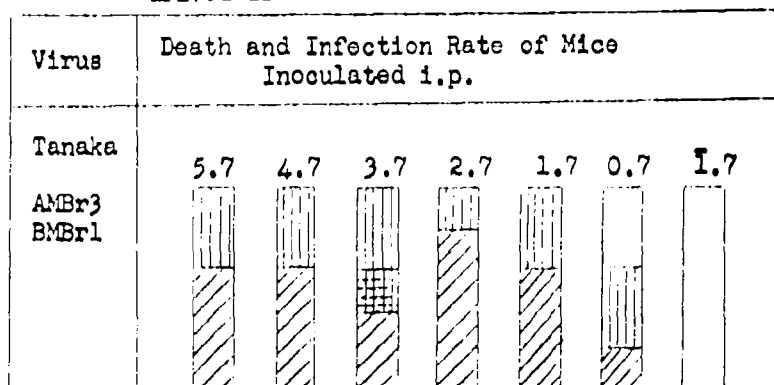
Others: Already stated in this report.

c. Experimental Results

1) Original character of Tanaka strain

As already reported by Okuno (1959), an infected human brain was emulsified and inoculated into one day old eggs on one hand and into adult mouse brain on the other. Standard passages were made from sick mouse brain twice through adult mouse brain and once through suckling mouse brain. At this passage level, peripheral infectivity test was made with adult mouse. Results are shown in Fig. 5. It is no doubt that Tanaka strain at earlier passage level was L^+I^+ type in peripheral infectivity to mice. It could infect adult mouse even with as low as 5 BLU.

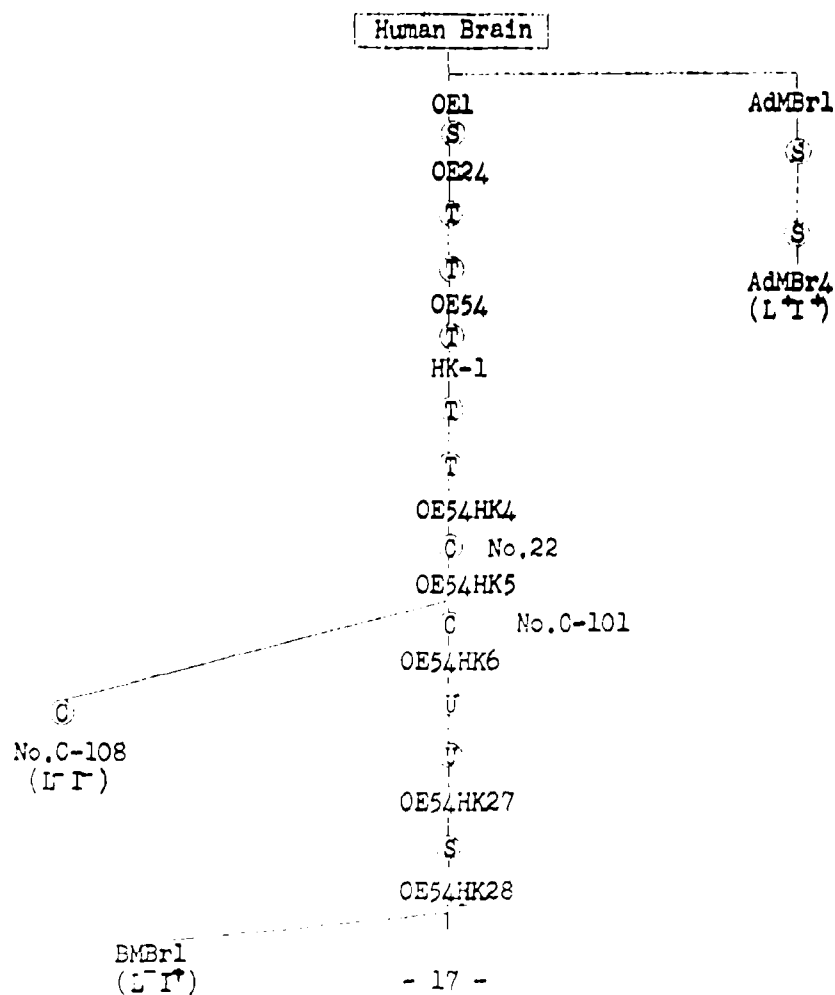
Fig. 5. Peripheral infectivity of strain
derived from Tanaka human brain.



2) Derived viruses of some attenuated characters of JBE strain.

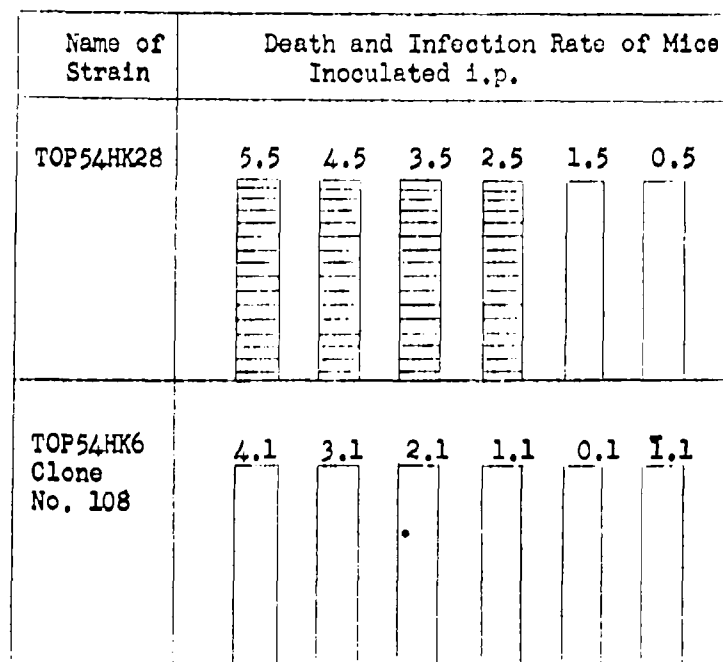
Attempting to select a virus strain of some attenuated virulence to mouse, passages were done employing one day eggs (OE) or HK cells as host and using various passage techniques. In Fig. 6 the whole passage history is illustrated.

Fig. 6. Passage History of TOP Strain



A clone (Cl.No.108) was selected from OE54HK5 and with a virus suspension of OE54HK28BM1 their peripheral infectivity were examined. Results are figured in Fig. 7.

Fig. 7. Peripheral infectivity of strains driven from the attenuated TOP strain



Note: The same to Fig. 1.

It is obvious that the virus of Cl.No.108 was completely L⁻I⁻ type, whereas the virus of OE54HK28BM1 had some L⁺I⁺ character stimulating antibody to the mouse.

As for the virulence by intracerebral inoculation to adult mouse, experiments were made at OE54HK27 level. Eight viral clones were selected from infected tissue culture fluid at OE54HK27. They were titrated with the adult mouse brain in parallel with HK cell. As shown on Table 7, the virus of this passage level was shown to have some attenuated character even with i.c. inoculation, though the interclonal variation was still remarkable.

Table 7 Virulence of TOP54HK27 clones to adult mice inoculated i.c.

Clone No.	Titer in log per cc with HK tubes	Mortality of mice inoculated 0.03 cc i.c. with a dilution of virus suspension						
		10 ⁻⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	
No. 2	5.0	2/5	3/5	5/5	0/5	1/5	0/5	
No. 3	5.0	3/5	3/5	2/5	2/5	0/4	0/5	
No. 5	5.0	3/5	4/5	3/5	1/5	0/5	0/5	
No. 8	<4.5	2/5	2/5	2/5	0/5	0/5	0/5	
No. 9	6.0	1/5	2/5	1/5	3/5	0/5	0/5	
No.10	7.0	3/5	3/5	2/5	1/5	1/5	0/5	
No.11	5.4	5/5	3/5	3/5	0/5	0/5	0/5	
No.13	<5.0	5/5	5/5	3/5	3/4	0/5	0/5	

d. Discussion

The present report clearly indicated that 3 lines of virus of distinct peripheral infectivity type were derived from a single starting material, an infected human brain. This fact support the idea that peripheral infectivity character may change during the random laboratory passages. Therefore, strain difference of peripheral infectivity previously described among a number of JBE strains maintained in the laboratory are reasonably explained.

It is still doubtful whether these changes occur during the passages or the difference existed in situ in nature. Prince (1960) reported a result which indicated heterogeneity of viral population of a strain concerning the peripheral infectivity to adult mouse. There seem to be good evidences that peripheral infectivity is controlled by genes but easily variable by passages.

As a consequence, our present investigation revealed a strain of JBE virus having L-I⁺ character which give us some hope to find a JBE strain which is completely avirulent to man as well as animals. Our attenuated strain was shown to be well attenuated not to kill the adult mouse by peripheral injection but still immunogenic by single injection to mouse. However, when it was applied directly into brain, it still retained some virulence though it was remarkably decreased. When our final purpose to apply it to man as a living vaccine is considered, the present virus is not satisfactory yet. Further effort should be needed to obtain a virus completely lacking of its virulence to brain tissue.

e. Summary

Starting from a human infected brain, 3 lines of virus bearing different peripheral infectivity were obtained. A strain, carrying L-I⁺ character, gave us some hope to get a strain completely avirulent to man which can be applied as a living JBE vaccine.

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5. ABSTRACT OF FINAL REPORT

Three strains of JBE virus were found to have characteristic peripheral infectivity to adult mice each distinct from the other two. Three infectivity types were designated as L^+I^+ , L^-I^+ and L^-I^- , being the first lethal and infective, the second nonlethal but infective and the third non lethal and non infective. The difference appeared to be highly reproducible and controlled by genes. A number of JBE virus strains maintained in our laboratory could be classified to one of those 3 types. General trend was noticed that newly isolates belonged to L^+I^+ type and laboratory-fixed strain to L^-I^- . An attempt was made to follow variation of peripheral infectivity during the experimental passages. Consequently, all three types were found to be segregated from a single source. A special concern was made for L^-I^+ type strain of JBE virus due to its applicability to a living vaccine to man.

Analytical examinations of growth pattern of typical L^+I^+ , L^-I^+ and L^-I^- viruses in the adult mouse inoculated peripherally revealed the theoretical background to the classification. It was also confirmed that the standardization of the host factors was important to apply the peripheral infectivity test.

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